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Commissioner for Patents
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DECLARATON UNDER 37 C.F.R. §1.132

I, Stephen B. Olmsted, hereby declare as follows:

(1) I am a researcher at Wyeth, which is the licensee of the above-identified application. I received my Ph.D. in Immunology from Cornell University, and held two post-doctoral fellowships at the University of Minnesota. Since then I have worked in the area of microbial vaccines with Wyeth. My career research interests have focused on bacterial pathogenesis and streptococcal genetics. I am currently Project Leader for a group A streptococcal vaccine which includes the claimed Streptococcal C5a peptidase (SCP) vaccine.

(2) We prepared an SCP double mutant and tested it for effectiveness as a protective vaccine. The two mutations in the SCP were directed to the aspartic acid at amino acid position 130, also called $Asp^{130}$ (where the numbering scheme follows the numbering of a full-length SCP that includes the signal sequence), and the serine at amino acid position 512, also called $Ser^{512}$. Both the $Asp^{130}$ and the $Ser^{512}$ were replaced with an alanine residue. Additionally, an SCP gene was isolated from a second bacterial background, a group B streptococcus, and identical mutations introduced at residues $Asp^{130}$ and $Ser^{512}$. Both versions of the inactivated

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SCP have been tested in an art recognized animal model and found to be protective not only against the homologous organism from which it was originally isolated, but also against the heterologous organism. Details of these findings can be found in the attached manuscript.

(3) I hereby declare that all statements made herein are of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

09-09-2004
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# Immunization with C5a peptidase from either group A or B streptococci enhances clearance of group A streptococci from intranasally infected mice

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## Abstract

Group A streptococci (*S. pyogenes*) are responsible for pharyngitis, impetigo and several more serious diseases. Emergence of toxic shock, and necrotizing fasciitis, associated with this pathogen over the past 10 years, has generated interest in development of a vaccine, which would prevent infections and potential serious complications. The highly conserved C5a peptidase that is expressed on the surface of group A streptococcus and other streptococcal species, associated with human infections, is a prime vaccine candidate. Here, we report construction of an inactive form of the peptidase and test its potential to induce protection in mice from intranasal challenge with either serotype M1 and M49 strains of streptococci. Mice were immunized by subcutaneous administration of recombinant proteins, mixed with Alum and monophosphoryl lipid A (MPL) adjuvants. Control mice were vaccinated with tetanus toxoid in the same adjuvants. Preparations of SCPA protein were highly immunogenic in mice. Antibody directed against protein from either group A (SCPAw) or group B (SCPBw) streptococci neutralized activity associated with both enzymes. Streptococci were cleared from the oral-nasal mucosa of mice immunized with vaccine protein more rapidly than those immunized with tetanus toxoid. Moreover, immunization with either protein enhanced clearance of group A streptococci from the lung. These results suggest that parenteral vaccination with SCPBw protein will provide protection against infection by either group A or B streptococci.


*Keywords:* Immunization; C5a peptidase; Streptococci

## 1. Introduction

Group A streptococci are responsible for a variety of human infections and complications. Streptococcal pharyngitis is alleged to account for 10–30% of office visits to a general medical practice. Although rheumatic fever continues to be common and responsible for the bulk of heart valve disease in children of the third world, vaccine development was ignored. In developed countries Streptococcal pharyngitis was considered relatively harmless and readily treatable with antibiotics. This perception changed in the early 1990s, when reports of serious group A streptococcal infections associated with toxic shock and necrotizing fasciitis became more common in the US and Europe. Moreover, clusters of rheumatic fever and sporadic cases of puerperal sepsis were also on the rise [1,2]. At the same time, it became apparent that penicillin failed to eradicate streptococci from the throats of up to 40% of children who are treated, and at least a third of those go on to have recurrent disease [3,4]. After 4 years of age children may have recurrent episodes of pharyngitis and tonsillitis. Österlund et al. reported that children who suffer from recurrent tonsillitis harbor intracellular streptococci that have avoided extensive penicillin therapy and suggested that tonsils can be the reservoir for recurrent disease [5].

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The goal of vaccine development is to prevent pharyngitis, thereby reducing or eliminating the reservoir for this common childhood disease. Elimination of pharyngitis and impetigo is presumed to ultimately reduce the incidence of more serious streptococcal infections, and reduce the potential for complications. Earlier attempts to develop group A streptococcus vaccines focused on the M protein [6,7], but safety considerations and recognition of antigenic variability in M proteins temporarily challenged this approach. More than 100 serotypes, determined by the antigenic variability of M protein, are known to exist and unlike pneumococcus and haemophilus disease can not be attributed to a limited number of serotypes. Instead the predominant serotypes in a population rapidly change, and vary considerably in different parts of the world.

Studies of streptococcal pathogenesis have revealed other virulence factors that are potential vaccine candidates. Our approach has focused on the C5a peptidase, an enzyme that is bound to the surface of most group A streptococcal strains tested and is antigenically conserved among different serotypes. This large protein antigen is also produced by human isolates of group C streptococci (unpublished data), group B [6,35], and G streptococci [7]. Experiments in mice showed that SCPA retards the influx of phagocytic cells and clearance of streptococci from subdermal sites of infection [8]. It was also shown to augment persistence of streptococci on the oral mucosa following intranasal infection [9]. Intranasal immunization initiated a specific IgA response and increased clearance of streptococci from intranasally infected mice [10]. Local immunization provided protection across serotype boundaries, as expected. The human immunological response to SCPA has not been extensively studied. O'Connor et al., however, discovered that most adults (79%) have measurable SCPA specific IgA in their saliva; whereas, fewer than 10% of children under the age of 10 years have measurable antibody [11]. The concentration of anti-SCPA IgA increases following pharyngitis [11]. Serum levels of anti-SCPA parallel those of anti-streptolysin O and peak between 10 and 12 years old [11,13].

The above observations suggested that SCPA could be used in a vaccine to prevent human disease. We propose that immunization with recombinant, enzymatically inactive SCPA would induce neutralizing antibodies that would augment the phagocytic response at the earliest stage of infection by preserving the C5a chemotaxin. This would in turn enhance clearance of streptococci before colonization is achieved. Here we describe construction of a mutant, enzymatically inactive form of peptidase in which the catalytic aspartate ($D^{130}$) and serine ($S^{512}$) were replaced with alanine. This protein was mixed with alum and MPL as adjuvants and injected subcutaneously into mice. Both the total specific antibody and neutralizing antibody response was evaluated. Experiments demonstrate that vaccination with C5a peptidase from either group A or group B streptococci induces an immune response that enhances clearance of streptococci from the lungs and nasal mucosa of intranasally challenged mice.

## 2. Methods

### 2.1. Bacterial strains and growth

Group A streptococcal strains 90-131, 90-226 [12,14] and MGAS5005 [15] are serotype M1, serum opacity negative and represent the highly invasive clone previously described. MGAS5005 was a gift of James Musser, Rocky Mountain Laboratory, Hamilton, Mt. Strain CS101 is an M49, serum opacity positive culture, which was previously described [16]. Streptococci were grown in Todd–Hewitt broth supplemented with 2% neopeptone (Difco Laboratories, Detroit, MI) or on solid media containing Difco Blood agar base and sheep blood cells. Overnight cultures were transferred into Todd–Hewitt broth that contained 20% rabbit serum (Gibco Life Technologies) and grown to optical density at 560 nm of 0.5–0.6 or log phase. Bacteria were washed three times with phosphate-buffer-saline (PBS) and resuspended in PBS so that 20 μl contained $1 \times 10^8$ to $10^9$ CFU/ml.

The *E. coli* strain Top10F (Invitrogen Corporation, Carlsbad, CA) was used for plasmid maintenance and was cultured in Luria-Bertani medium. BL21(DE3) (Novagen, Inc., Madison, WI) was used for protein expression and was cultured in 20 g/L HySoy (DMV International, New York, NY), 5 g/L yeast extract (Difco), 0.5 g/L sodium chloride, and 10 mM potassium phosphate buffer (pH 7.2). Antibiotics were used at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), and chloramphenicol (30 μg/ml). Restriction enzymes were obtained from New England Biolabs.

### 2.2. Construction of ΔSCPA49, SCPAw and SCPBw recombinant vaccine proteins

An enzymatically inactive, truncated form of SCPA49 protein, ΔSCPA49, was produced in *E. coli* and purified by affinity chromatography as previously described [8]. SCPAw originated from the wild-type *scpA* gene that was amplified by PCR from the M1 serotype 90-226 strain of streptococcus in the following manner. A segment of the gene from just after the signal sequence to the beginning of the cell wall repeats (bases 94-3112) was amplified by PCR using the forward primer 5′-CCC GAA TTC AAT ACT GTG ACA GAA GAC ACT CCT GC-3′ and the reverse primer 5′-CCC GGA TCC TTA TTG TTC TGG TTT ATT AGA GTG GCC-3′. Using the *Eco*RI engineered site at the 5′ end and the *Bam*HI site engineered at the 3′ end, the fragment was cloned into pTrc99a (Amersham Pharmacia Biotech, Piscataway, NJ), placing the C terminal truncated product in frame with the ATG start codon of the vector. This construct, plasmid pLP605, was transformed into *E. coli* DH5α cells for expression of wild-type active peptidase.

Additional modifications included the deletion of the 60 amino acid propeptide at the N-terminus [17] by PCR and inactivation of two out of three catalytic residues. Site-specific mutagenesis of the active aspartate at position 130 and the

serine residue at position 512 in the wild-type protein was done following a procedure described by Fisher and Pei [18]. Briefly, primers were designed for inverse PCR such that the two would abut each other in opposite orientations, and contained codon changes that replaced the aspartate and serine to alanine. Separate, inverse PCR amplification of plasmid pLP605 with mutant primers was performed using Expand™ Long Template PCR System (Boehringer Mannheim Corporation, Indianapolis, IN) resulting in a blunt end product. The PCR product was self-ligated and transformed into *E. coli* TOP10F′ (Invitrogen Corporation, Carlsbad, CA). Once clones of interest were confirmed by DNA sequencing, the two mutations were combined by ligation of restriction fragments. The *scpA* D130A/S512A fragment from the resultant mutant plasmid, pLP664, was finally excised by restriction digestion and subcloned into plasmid pBAD18, resulting in plasmid pLP672, which was transformed into the *E. coli* strain BLR for expression.

Generation of the C5a peptidase from group B streptococcus was conducted in a manner similar to that described for the group A streptococcal C5a peptidase. Because the nucleotide sequences of *scpB* and *scpA* to be amplified and subcloned are nearly identical the same primers were used for both. The DNA template used for cloning of wild-type *scpB* was from the serotype II strain 78-471 of group B streptococcus. The final *scpB* D130A/S512A double mutant was cloned into plasmid pBAD18 to produce plasmid pLP676, which was also transformed into *E. coli* strain BLR for expression.

### 2.3. Purification of recombinant D130A/S512A C5a peptidase (SCPAw and SCPBw)

An overnight culture of either *E. coli* BLR(pLP672) or BLR(pLP676) was diluted 1:100 in fresh HSY medium containing chloramphenicol and grown to an $OD_{600}$ equal to 4.0 before induction with 1% arabinose. Both proteins were purified by a combination of ammonium sulfate precipitation and ion-exchange chromatography on Q-Sepharose as previously described [17]. Purified recombinant C5a peptidases were stored frozen at −20 °C.

### 2.4. Evaluation of C5a peptidase enzymatic activity

Enzymatic activities of wild type C5a peptidase and D130A/S512A mutants were evaluated using the chromogenic substrate Ac-SQLRANISH-pNA [17]. For this purpose, 59 μg (1 μM) wild-type C5a peptidase and Ac-SQLRANISH-pNA (110 μM) substrate were incubated at 37 °C in 100 mM Tris, pH 8.6. Analyses of D130A/S512A mutant proteins were performed with 2.1 mg (33 μM) of protein. In both cases, the total volume of the reaction mixture was 0.5 ml. Assays were carried out in 1 cm path-length quartz cells. Reactions were monitored by continuous measurements of absorbance at 405 nm for 180–900 min. using a Spectromic Genesys 2 Spectrophotometer [17].

### 2.5. Immunization protocol

Vaccines containing tetanus toxoid (TT), ΔSCPA49, SCPAw and SCPBw proteins were prepared by mixing 5 μg of antigen with 100 μg of AlPO4 (Alum) in a 50 μl volume overnight at 4 °C. The following day 50 μg of MPL (RIBI Immunochem Research, Hamilton, Mt.) was added, resulting in a total volume of 100 μl. The combination of Alum and MPL was used to achieve a more robust and broader IgG isotype response to SCPA protein. Four-week-old female CD1 mice (Charles River Laboratories) were immunized subcutaneously at the scruff of their neck with 5 μg of antigen per dose. Mice were boosted at 4 and 6 weeks following the first inoculation with the same amount of antigen in adjuvant. Mice were bled after 10 days and challenged with streptococci 13 days after the last boost.

### 2.6. Antibody assays

Mouse anti-SCPA IgG was measured by ELISA using 250 ng of SCPAw to coat wells as previously described [19]. Goat anti-mouse IgG conjugated to alkaline phosphatase was used as the secondary antibody. Plates were developed for 30 min after addition of substrate, titers are reported as reciprocal dilutions that resulted in an absorbance, two standard deviations greater than negative controls (sera from mice immunized with TT).

Neutralizing titers were assessed by measuring residual C5a peptidase activity, following incubation of peptidase with sera, and using a GST-C5a-GFP substrate [20]. One hundred microlitres samples of sera diluted in PBS/1% BSA were added to 250 ng of SCPA and the mixtures were incubated at 37 °C for 2 h (neutralization of enzyme with antibody). Then 20 μl of 50% GST-hC5a-GFP sepharose beads [20] were added to the SCPA–serum mixture, which was incubated for 45 min at room temperature. Released GFP was measured with a Bio-Tek FL600 Fluorescence reader. Neutralization titers were extrapolated from the dilutions that inhibited 50% of the C5a peptidase activity.

### 2.7. Hep2 cell and fibronectin binding assays

Protein binding to either Hep2 cells or fibronectin was measured using an ELISA format as previous described [21].

### 2.8. Infection of mice

Experimental groups consisted of 8–10-week-old mice (8–19 mice per group). Animals were anesthetized with isoflurane before inoculation with 20 μl of bacterial suspension, 10 μl into each nare. At various times after infection mice were euthanized by asphyxiation with $CO_2$, and nasal tissue was harvested en bloc. Following disinfection with 70% ethanol the skin on the snout was removed. Nasal tissue was sampled with dog nail clippers placed over the maxilla, just before the eyes. Tissue samples were added to 2 ml of

saline and weighed. Tissue was then homogenized, and cultured quantitatively on Columbia blood agar plates. Columbia agar is a selective medium, which limits growth of other bacteria contaminating homogenized tissue preparation. After overnight incubation at 37 °C, the number of β-hemolytic colonies on plates was counted. The quantity of CFU/mg tissue was calculated and the geometric mean for each group of mice was calculated.

## 3. Results

### 3.1. Vaccine constructs

A truncated form of the peptidase, SCPAw, which lacked the signal sequence, propeptide, cell wall spanning region and peptidoglycan anchor was constructed by subcloning the appropriate PCR fragment using total DNA from strain 90 to 226, a serotype M1 strain as template (Fig. 1). Residues, aspartic acid ($D^{130}$) and serine ($S^{512}$), were replaced with alanine to inactivate protease activity. SCPBw was derived from group B streptococcus, strain 78-491, a serotype II culture [22]. Enzymatic activity of recombinant wild type peptidase and the effects of amino acid substitutions were assessed using chromogenic substrate Ac-SQLRANISH-pNA, which corresponds to a segment of human C5a. As expected, incubation of Ac-SQLRANISH-pNA in the presence of wild type C5a peptidase from group A streptococci was accompanied by an increase of absorbance at 405 nm, due to enzymatic release of *p*-nitroamiline (Fig. 2). In contrast incubation of C5a peptidase D130A/S512A mutant proteins did not result in hydrolysis of substrate, indicating that the amino acid substitutions eliminated peptidase activity. Activity of the mutants was not detected even after prolonged incubation for 900 min with the Ac-SQLRANISH-pNA substrate.

SCPA and SCPB proteins were recently discovered to be bi-functional; both proteins bind to fibronectin and epithelial cells with relatively high affinity [21]. The impact of these binding activities on streptococcal pathogenesis is unknown,

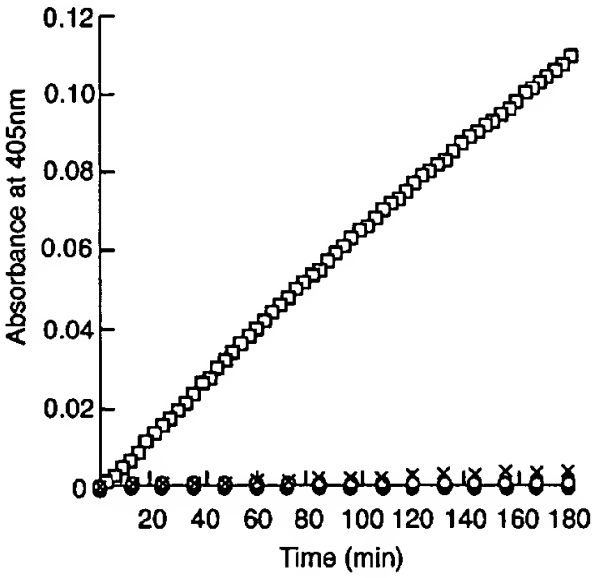


Fig. 2. Comparison of the protease activity associated with wild type SCPA (□), SCPAw (●) SCPBw (○) proteins and no enzyme (×). 0.5 ml reaction mixtures contained 59 μg (1 μM) wild type C5a peptidase and Ac-SQLRANISH-pNA (110 μM) substrate in 100 mM Tris, pH 8.6 and were incubated at 37 °C. Mutant protein assays contained 2.1 mg (33 μM) of protein. The blank contained 110 μM substrate without added enzyme.

therefore, it was of interest to know whether mutant proteins used in this study retained ability to bind to Hep2 cells and to fibronectin. As shown in Fig. 3, deletion of the N-terminal region, containing the signal and propeptide sequences, and the C-terminal cell wall spanning-anchor domain dramatically reduced binding to both fibronectin and epithelial cells.

### 3.2. Subcutaneous immunization of mice with SCPA and adjuvants induces a strong serum antibody response

In earlier studies, we reported that intranasal inoculation of mice with purified recombinant ΔSCPA49, originally from an M49 strain, induced measurable SCPA specific sIgA in saliva, and produced serotype independent protection from intranasally administered streptococci [10]. In the following experiments, we tested whether subcutaneous (SC) immunization of mice with recombinant SCPAw mixed with Alum

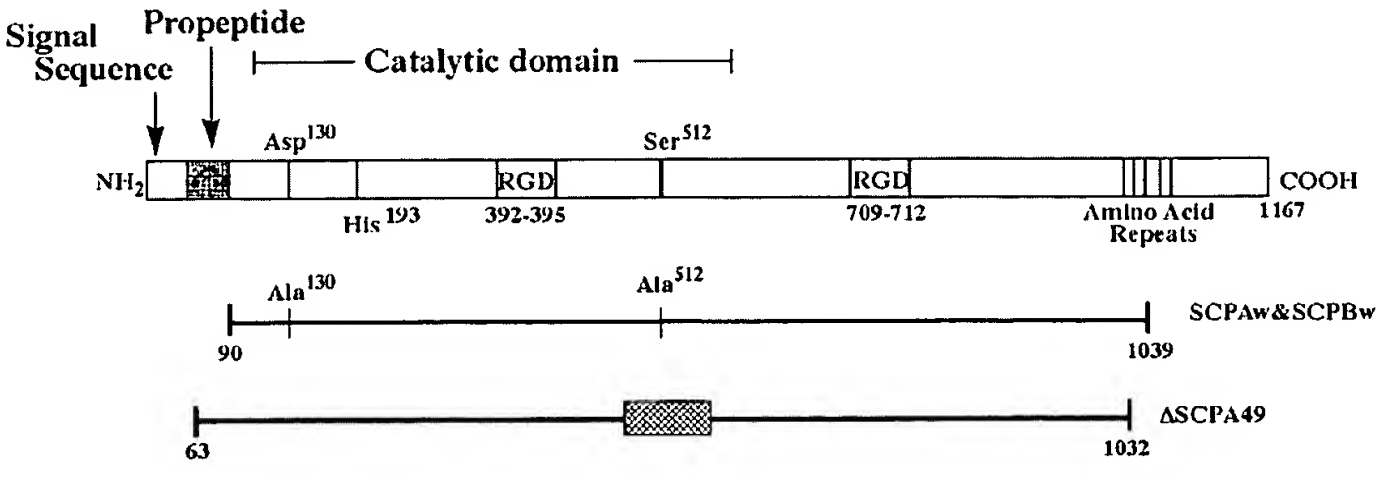


Fig. 1. Functional map of the streptococcal C5a peptidase. The first 31 amino acids correspond to the signal sequence, residues 32–59 correspond to the propeptide which is removed by autocatalytic intramolecular cleavage; $Asp^{130}$, $His^{193}$, and $Ser^{512}$ compose charge transfer residues, required for protease activity; RGDs (arginine, glycine, aspartic acid) sequences are located at positions 395 and 712. The solid heavy line represents the SCPAw and SCPBw forms of the protein with alanine substitution at positions $Asp^{130}$ and $Ser^{512}$. The hatched box shows the position of deleted amino acids.

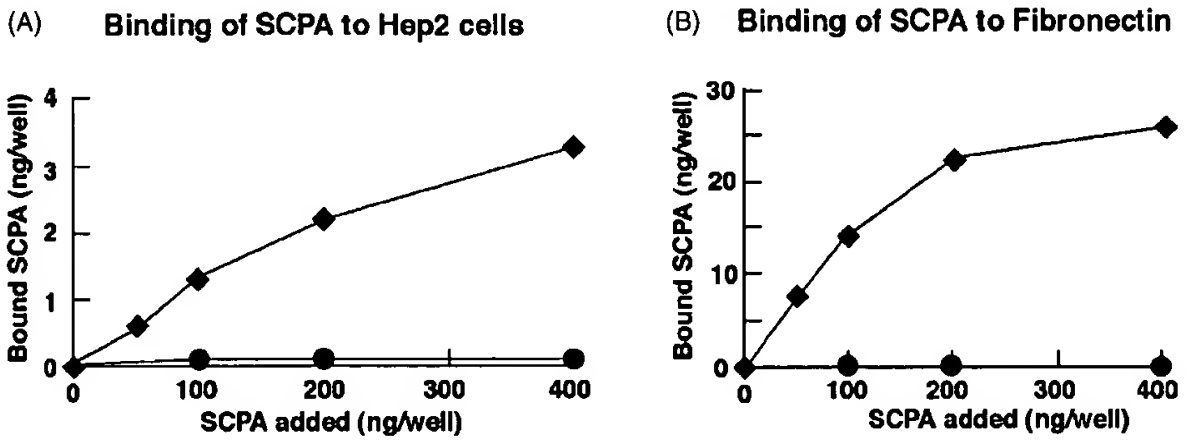


Fig. 3. Binding of SCPA protein to Hep2 cells and soluble fibronectin: (♦) activity of wild type full-length SCPA protein, (●) activity of the vaccine protein SCPAw. Purified protein was incubated with Hep2 cells (A), or Fibronectin coated microtiter plates (B) for 2 h at 37 °C. Bound SCPAw was detected with rabbit anti-SCPA, followed by HRP-goat anti-rabbit antibody, standard curves were developed by binding known amounts of protein to Maxisorb microtiter plates. Standard errors were less than 8% for all experiments.

and MPL adjuvants would also produce a strong antibody response, which would increase clearance of streptococci from the nasopharynx of mice. As expected, SC delivery of SCPAw antigen resulted in a strong serum antibody response. Titers of specific anti-SCPA IgG from two independent experiments are shown in Fig. 4. Mean ELISA titers approximated 1/64,000 and ranged from 1/25,000 to 1/265,000.

We presumed that neutralizing antibody would enhance infiltration of phagocytes and be important for protection against infection. Neutralizing antibody titers were determined using an assay based on cleavage of the fluorescent C5a mimic substrate, GST-hC5a-GFP [20]. Dilutions of mouse sera were pre-incubated with SCPA wild type, affinity purified enzyme, and then residual peptidase activity was quantitated fluorometrically by release of GFP. The extrapolated dilution of serum that inhibited 50% of the SCPA activity

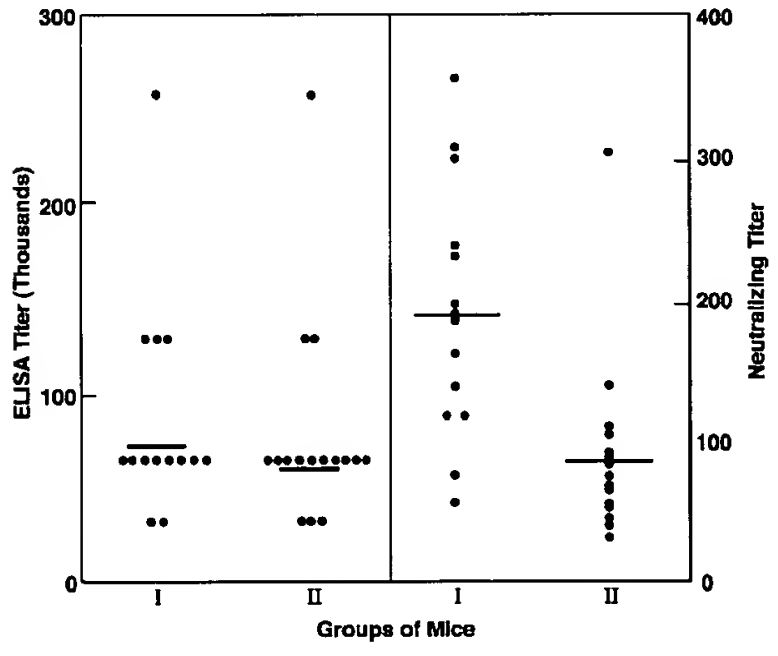


Fig. 4. Comparison of total anti-SCPA and neutralizing antibody titers of sera from two groups of mice, vaccinated at different times, with different batches of antigen. The vaccination protocol and assays are described in the methods section. Horizontal bars define the geometric mean of total antibody titers and the arithmetic mean of neutralizing titers.

defined the titer (Fig. 4). Mouse sera had titers, which ranged from 1/20 to 1/375. Like total specific IgG titers, neutralizing antibody levels varied considerably from animal to animal. None of the mice that were immunized with tetanus toxoid had measurable anti-SCPA antibodies. The lower mean neutralizing titer in group II mice was a concern; therefore, assays of both sets of sera were repeated using a single preparation of GST-hC5a-GFP substrate. Similar differences in magnitude of titers were again observed (data not shown). Differences from experiment to experiment most likely reflect variations in mice, but could also be due to unknown differences in the quality of antigen lots. Analysis of sera from two experiments with 16 mice in each experimental group showed that neutralizing titers correlated positively with the magnitude of ELISA titer. The correlation coefficient for group I was 0.67 with a $P = 0.006$ and 0.83 with $P = 0.0001$ for the second group of mice. Neutralizing titers, however, did not significantly correlate with protection. We are not certain whether this discordance reflects limits in resolving power of protection experiments, or whether neutralizing antibodies in mice are less important for protection than anticipated.

### 3.3. Protection studies

An important objective of this study was to determine whether a parenteral route of vaccination would enhance clearance of streptococci from the nasopharyngeal mucosa and/or prevent colonization of mice. In the first experiment, throats of intranasally infected mice were swabbed daily with caliginate swabs. Swabs were streaked on sheep blood Columbia agar plates. A swab that produced more than two colonies on a plate was considered a positive culture. Mice were immunized as described in the methods section with either SCPAw or ΔSCPA49 antigen, originally derived from serotype M1 and M49 streptococci, respectively. Control mice were immunized with tetanus toxoid and adjuvants. Three separated experiments were performed. In the first, experiment I, mice immunized with ΔSCPA49 protein and adjuvant more completely cleared M49 streptococci from their

Table 1
Clearance of streptococci from the oral-pharynx of intranasally infected mice

| Vaccine antigen | Challenge[a] strain | Percent positive cultures[b] | |
|---|---|---|---|
| | | Tetanus toxoid | SCPA protein |
| Experiment I: ΔSCPA49 | M49 CS101 | 57.8 (19) | 8.3 (12) |
| Experiment II: ΔSCPA49 | M1 90-131 | 33.3 (15) | 8.3 (12) |
| Experiment III: SCPAw | M1 90-131 | 23.1 (13) | 7.6 (13) |

[a] Experimental groups varied in number of mice. M49 and M1 designate the serotype. Mice were inoculated intranasally with approximately $1.5 \times 10^9$ CFU.

[b] Percent positive cultures is the number of mice with positive cultures/number of mice inoculated. Numbers in parentheses equal the number of mice in each experimental group. Data are from specimens taken on days 4 and 5 for mice inoculated with strains CS101 and 90-131, respectively. The difference between mice immunized with tetanus toxoid and SCPA proteins were evaluated by the Fisher Exact Test; $P = 0.01$, 0.16 and 0.59 for experiments I, II, and III, respectively.

throats than those immunized with tetanus toxoid and adjuvants (Table 1). Differences were statistically significant ($P = 0.01$) in this experiment. In two other experiments mice were immunized with either ΔSCPA49 or SCPAw protein with adjuvants. These mice cleared serotype M1 streptococci more completely from their throats than control mice; however, differences were not statistically significant. Although a trend for SCPA vaccinated mice to clear streptococci more efficiently than control mice was consistent with previous studies [10], lack of reproducibility was bothersome and prompted us to try another method to assess persistence of streptococci in immunized animals.

To reduce variability associated with throat cultures and reassess protection we quantitated CFU in excised, homogenized nasal tissue as described in the methods. At various times following intranasal inoculation mice were euthanized, and then nasal turbinates and associated lymphoid tissues were removed and homogenized in PBS buffer. Viable counts were performed by plating samples of homogenates on Columbia sheep blood agar. In the first experiment groups of mice were vaccinated with either SCPAw or tetanus toxoid and infected intranasally with the M49 strain CS101. CFU associated with dissected nasal tissues from three groups of mice at 16, 24, and 48 h post-infection were determined. As expected the number of CFU/mg tissue decreased with increasing time (Fig. 5). At 16 and 24 h post-infection mice that had been vaccinated with SCPAw had significantly fewer

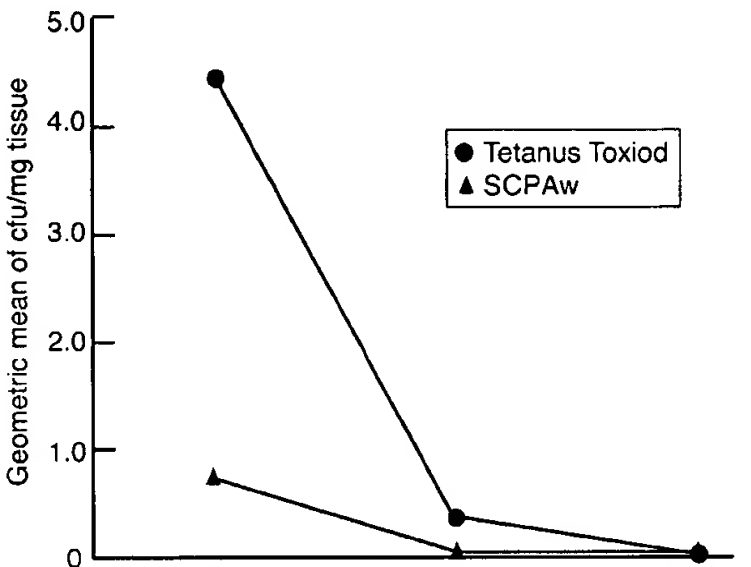


Fig. 5. Residual CFU of GAS associated with homogenized nasal tissue: (●) the geometric mean number of CFU associated with nasal tissue dissected from mice immunized with tetanus toxoid and adjuvants, (▲) the geometric mean number of CFU associated with nasal tissue dissected from mice immunized with SCPAw protein, mixed with Alum and MPL adjuvants. Mice were challenged by intranasal inoculation with $1 \times 10^8$ CFU. Two-way ANOVA comparisons confirmed that the overall rate of clearance of streptococci from mice immunized with SCPAw protein was significantly different from the rate of clearance from mice immunized with tetanus toxoid. *P*-value for this difference is 0.02.

Table 2
Clearance of streptococci from nasal tissue following intranasal inoculation

| Challenge strain | Number of mice | Geometric mean of CFU per 10 mg of tissue | |
|---|---|---|---|
| | | Vaccine tetanus toxoid | Vaccine SCPAw |
| Experiment I: M1 90226[a] | 14 | 30.0 | 0.0 |
| Experiment II: M1 90226[b] | 16 | 330.0 | 39 |
| Experiment III: M49 CS101[c] | 8 | 230.0 | 13 |

[a] Mice were challenged intranasally with $6.5 \times 10^4$ CFU. Twenty hours after infection they were euthanized and nasal tissues obtained. Differences between mice immunized with tetanus toxoid and SCPAw were determined to be statistically significant with $P = 0.02$, using the Wilcoxon two-side test. Total and neutralization titers for mice in experiments I and II are shown in Fig. 4.

[b] Mice were challenged with $1.2 \times 10^5$ CFU. Nasal tissues were obtained after 16 h. Differences between mice immunized with tetanus toxoid and SCPAw were determined to be statically significant with $P = 0.001$, using the Wilcoxon two-side test.

[c] Mice were challenged intranasally with $6.0 \times 10^7$ CFU. Mice were euthanized 24 h. after infection and tissues were obtained. Differences between mice immunized with tetanus toxoid and SCPAw were determined to statically significant with $P = 0.001$ using a two-way ANOVA test.

CFU/mg tissue than those immunized with tetanus toxoid. Differences were not significant at the 48 h time point because one control mouse had died and the rest were nearly free of viable streptococci. After removing the time variable in the analysis and combining data from all three groups, a Two-way ANOVA comparison confirmed that the overall rate of clearance of streptococci from mice immunized with SCPAw protein was significantly greater than the rate of clearance from mice immunized with tetanus toxoid. *P*-value for this difference is 0.02. Note that smaller doses of streptococci were used in this and subsequent experiments than in the earlier experiment where throat swabs were used to assess residual streptococci.

Subsequent experiments tested the influence of immunization on clearance of a serotype M1 strain 90-226 by quantitating CFU present in excised nasal tissue at a single time point after intranasal infection (Table 2). Both total SCPA specific antibodies and serum protease neutralizing titers varied significantly between mice (Fig. 4). In each experiment, mice vaccinated with SCPAw protein had more effectively eliminated streptococci from nasal tissue, than those vaccinated with tetanus toxoid. Moreover, although the *scpA* gene originated from a serotype M1 strain, the SCPAw protein induced protection against both M1 and M49 challenge strains of streptococcus, confirming that protection crosses serotype boundaries.

### 3.4. Antibody directed against SCPA or SCPB neutralizes C5ase activity

The sequence of *scpA* and *scpB* genes and the proteins they encode are 95–98% identical [22,23]. Antibody directed against either protein will react with both proteins on Western blots, and the proteins equally compete with each other in competitive ELISA experiments (data not shown). These similarities suggest that antibodies to either protein should neutralize cleavage of C5a and that vaccination with either protein should induce a protective immune response. Experiments were performed to determine whether rabbit antibody, induced by each protein, is able to neutralize the C5ase activity of the other protein. In these experiments either recombinant, wild type SCPA or SCPB proteins were pre-incubated with rabbit serum. After 2 h, the amount of residual C5ase activity was measured using the GFP release assay (Table 3). Sera from rabbits immunized with either SCPAw or SCPBw protein significantly neutralized activity associated with both SCPA and SCPB enzymes. Differences in percent inhibition of SCPA and SCPB activities were not statistically significant. Serum from a rabbit immunized with tetanus toxoid had no effect on activity.

Neutralization of C5ase activity associated with intact group A and B streptococci was also tested (Table 4). Antiserum directed against SCPAw inhibited from 38.2 to 73.3% of the GFP released by either genus of streptococcus during 16 h incubation of bacteria with GST-C5a-GFP bound to Sepharose beads. Rabbit anti-SCPBw also inhibited from

Table 3
Rabbit antibody inhibits both SCPA and SCPB C5a peptidase activity

| Enzyme serum | Percent inhibition | |
|---|---|---|
| | SCPA peptidase | SCPB peptidase |
| No serum | 0.0 | 0.0 |
| Rabbit anti-SCPA | 90.8 | 63.9 |
| Rabbit anti-SCPB | 91.1 | 59 |
| Rabbit anti-TT | 0.0 | 0.0 |

Anti-TT is serum from a rabbit immunized with tetanus toxoid. Dilutions (1/100) of sera were preincubated with 200 ng of purified recombinant protein in Tris-BSA buffer in a total volume of 100 μl for 2 h at 37 °C. Triplicate 20 μl samples were removed and mixed with 20 μl of a 50% suspension of GST-C5A-GFP beads. Relative fluorescence units (RFU) were measured after 45 min incubation at 37 °C. Percent inhibition was calculated from the formula: $[(RFU_{anti-TT\ serum} - RFU_{rabbit\ serum})/RFU_{anti-TT\ serum}] \times 100$. Percent inhibition is from a single representative experiment.

53.2 to 69% of the activity associated with group A strains with different M proteins and three group B strains of different serotype. Differences in percent inhibition between strains of streptococci or sera were not statistically significant. These results correlated with previous protection studies, which demonstrated enhanced clearance of group A streptococci from the nasopharynx of mice immunized with SCPA [10] and with those that observed clearance of group B streptococci from lungs of mice immunized with SCPB [19].

### 3.5. Immunization with SCPAw or SCPBw increases clearance of streptococci from lungs

The fact that hyper-immune rabbit serum directed at either SCPA and SCPB proteins neutralized enzymatic activity encouraged us to test whether immunization with either protein would enhance clearance of streptococci from lungs of mice. The infection model and conditions were the same as those used to investigate clearance of group B streptococcus from mouse lungs [19]. Mice were immunized as described above, but they were challenged intranasally with a relatively

Table 4
Antibody inhibition of C5a peptidase activity associate group A and B streptococcal cells

| Streptococcal strain[a] | Anti-SCPAw percent inhibition[b] | Anti-SCPBw percent inhibition |
|---|---|---|
| 90226 serotype M1 | 73.3 ± 0.9 | 60.0 ± 5.0 |
| CS101 serotype M49 | 42.1 ± 13.9 | 37.6 ± 21.8 |
| M5005 serotype M1 | 61.8 ± 11.6 | 53.1 ± 8.2 |
| GBS S2 serotype VI | 70.6 ± 1.1 | 68.5 ± 1.3 |
| GBS serotype III | 64.5 ± 5.0 | 44.5 ± 9.4 |
| 78-471 serotype II | 38.2 ± 27.4 | 53.2 ± 8.2 |

[a] Washed log phase bacteria, grown in THY, were concentrated to $5 \times 10^{10}$ ml$^{-1}$ in Tris-BSA buffer. Ninety microlitres of each suspension was mixed with a 1/50 dilution of rabbit antiserum and incubated 2 h at 37 °C before addition of 20 μl of beads with bound GST-C5a-GFP. This mixture was incubated 16 h at 37 °C. Bacterial cells and beads were pelleted by centrifugation and fluorescence retained in the supernatant was determined in triplicate.

[b] Percent inhibition of GFP release from beads is the mean of two independent experiments ± S.E.M.

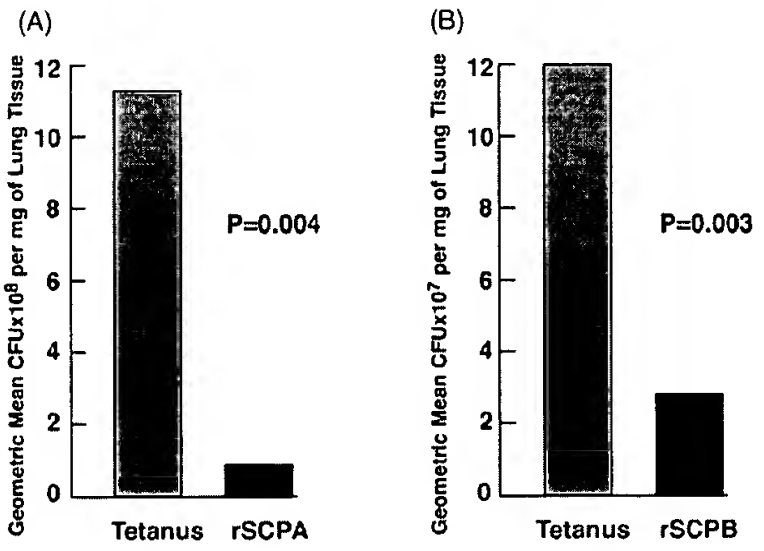


Fig. 6. Residual CFU of streptococci associated with homogenized lungs; grey bars are CFU cultured from lungs derived from mice immunized with tetanus toxoid, black bars are CFU cultured from lungs derived from mice immunized with SCPA (A), from mice immunized with SCPB (B). The two-sided exact *P*-values were calculated by the Wilcoxon rank-sum test.

larger volume of 90-226 group A streptococcus suspension. Instead of 20 μl, 50 μl of streptococcal suspension were introduced into one nostril, which results in reproducible introduction of streptococci into the lungs [19]. An effective vaccine should block infection at the earliest possible stage of infection before the bacteria have had an opportunity to colonize mucus membrane or establish infection at a normally sterile site. This consideration prompted us to assess vaccine efficacy shortly after inoculation. As observed in the nasopharynx most bacteria were cleared from the lungs by 48 h, irrespective of immune status. First, it was determined that 4–7 h. post-inoculation was an optimal time for 90% of infected control mice to retain measurable viable streptococci in their lungs (data not shown). Infection of mice, immunized with SCPAw protein significantly reduced the number of mice that retained streptococci and reduced the number of CFU associated with homogenized lung tissue (Fig. 6A). The difference in CFU in lung tissue between control mice and those immunized with SCPAw was significant with $P = 0.004$. Immunization with SCPBw had a comparable impact on clearance (Fig. 6B). Mice immunized with SCPBw also cleared streptococci from their lungs more efficiently than those immunized with tetanus toxoid. This difference had a $P = 0.003$. These experiments confirmed that immunization with C5a peptidase protein from either group A or group B streptococci will provide protection against group A streptococcus infections.

## 4. Discussion

Group A streptococcus is responsible for one in ten visits to a general practitioner's office, and streptococcal disease is even more frequent during fall and winter months in temperate climates from the United States to Europe. Although serious disease, such rheumatic fever, child bed sepsis and other debilitating and deadly infections declined dramatically in the 19th century, in the US and Europe this pathogen continues to be burdensome for families. What parent has not had to interrupt their work schedule several times a year to take a 5–10-year-old child to the pediatrician for throat cultures or one of several rapid tests for group A streptococcus. Moreover, 25–40% of the time oral penicillin fails to eradicate the bacteria, and among those failures nearly a third will have repeated infections by the same strain of streptococcus [3,4,24]. The incidence of serious streptococcal infections among the poor and disadvantaged in both developed and under developed countries has changed little over the past century. The incidence of rheumatic fever has been reported to be as high as 4–6/1000 in Indian and African populations, and among indigenous people of Australia and New Zealand [25,26]. In many economically deprived regions of the world 40% of those who visit cardiology clinics have mitral valve disease, a remnant of childhood rheumatic fever. The World Health Organization estimates that 500,000 die every year from Rheumatic fever or rheumatic heart disease, and those disabled by streptococcal disease are significantly greater in number [27].

An optimal vaccine would interrupt colonization of the throat by all serotypes and fail to induce autoimmune reactions and other potential side effects. Vaccinologists anticipate that significant reductions in pharyngitis and carriage of streptococcus would also greatly diminish serious streptococcal disease. Here, we investigated whether parenteral immunization, a widely employed, relatively safe method for vaccine delivery, would also produce protection against intranasal infection with group A streptococcus. The vaccine protein, SCPAw, contains a truncated form of the streptococcal C5a peptidase, which lacks propeptide and cell wall anchor domains, and which contains mutations in two amino acid residues required for catalytic activity. This enzymatically inactivated recombinant protein was mixed with Alum and MPL adjuvants just prior to subcutaneous injection. Together these adjuvants are expected to induce a more intense and broader IgG isotype immune response than either one alone.

Humans are the only known hosts for group A streptococcal infection. Primate, like mouse models, require relatively large numbers of group A streptococcus to produce long term colonization, and are prohibitively expensive. Therefore, we chose the murine intranasal infection model that had been previously employed to evaluate group A streptococcus virulence [8–10,19,29]. Some investigators have questioned the intranasal mouse model because mice lack palatine tonsils and because they are relatively resistant to infection. Moreover, variation in both cellular and extracellular virulence factors among different strains and serotypes requires optimization of the dose of streptococcus and time of specimen collection for each strain used in the study. Though rarely achieved, long-term colonization requires that strains be passed several times in mice. This was shown to dramatically change both cell surface and extracellular proteins ex-

pressed by the bacteria [30]; changes that may not be pertinent for persistence in human tonsils or the nasopharynx. In most experiments we assessed the impact of vaccination on clearance of streptococci from the oral-nasal mucosa very early after intranasal inoculation. Our rationale was that the most effective vaccine would promote immediate clearance of streptococci from the mucosa, before they have adjusted to this microenvironment, expanded their numbers and colonized. We presumed that colonization precedes symptoms and disease. Experiments with bioluminescent recombinant group A streptococcus validated our decision to assess retention of streptococci within hours after intranasal inoculation, and suggested that this model of infection may better reflect the human situation than originally anticipated [28]. Bioluminescent bacteria are initially cleared from the nasal mucosa, but their numbers increase again by 24 h with formation of microcolonies dispersed throughout nasal associated lymphoid tissue (NALT) [28].

Our goal here was to enhance clearance of streptococci from the nasopharyngeal mucosa. Since the challenge strains used in this study had not been passed in mice and produce relatively small hyaluronic acid capsules, control mice, immunized with tetanus toxoid, effectively cleared most streptococci within 48 h. Both throat cultures and viable counts of streptococci associated with homogenized nasal tissue were used to evaluate persistence of streptococci. The latter method was more quantitative than throat swabs, but was still subject to significant variation from animal to animal, and from experiment to experiment. In earlier experiments, nonanesthetized mice were inoculated intranasally; however, for later experiments mice were lightly anesthetized with isoflurane before inoculation with bacteria. This somewhat reduced variability between animals. We expect that variability also reflects the genetic dissimilarity of these outbred mice.

Subcutaneous injection of SCPAw protein with adjuvants clearly resulted in more rapid clearance of streptococci from the noses and throats of mice, whether residual streptococci were assessed by throat culture or quantitated in excised, homogenized suspensions of external nasal tissue. SCPA specific IgG titers were routinely high following vaccination, and the vaccine did not cause observable deleterious effects on mice. Although neutralizing antibody was measurable in immunized animal sera and titers had a positive correlation with total IgG titers, the quantity of neutralizing antibody did not correlate with protection. We suspect that the resolving powers of protection experiments and the protease assay are insufficient to make this correlation; however, other explanations are also possible. We recently discovered that SCPA and SCPB proteins bind directly to epithelial cells in vitro and contribute to invasion of these cells by both group A and B streptococci ([21], Cleary unpublished). Moreover, antibody directed against either protein deters ingest of both species of streptococci by these cells. Therefore, blockage of bacterial invasion of the nasal mucosa by anti-SCPA may be as important as neutralization of proteolytic activity in the experimental infection model used here.

Since SCPAw and SCPBw proteins are 98% identical in sequence we postulated that immunization with either protein would provide protection again group A streptococcal infections. Immunization of mice with SCPB promoted clearance of group B streptococci that are introduced into their lungs [19]. As predicted, vaccination of mice with SCP protein from either species resulted in high antibodies titers (data not shown), and more rapid clearance of a serotype M1 strain from their lungs. Thus, a vaccine that contains either SCPAw or SCPBw protein may reduce the incidence of disease caused by both common streptococcal pathogens. Although less common Group C, and G streptococci are associated with pharyngitis, as well as more serious systemic infections [29,31]. The DNAs from human isolates of these species also encode SCP proteins that are highly similar to SCPA ([7], unpublished data). Therefore, it is reasonable to postulate that immunization with a C5a peptidase subunit vaccine would also reduce the incidence of infections caused by these less common species.

Vaccine development for prevention of group A streptococcal disease has long focused on antigenically variable M protein. More recent efforts have attempted to circumvent antigenic variability and potential tissue cross reactive characteristics by using defined regions of recombinant M protein. Immunization with recombinant peptides, which contain the conserved C-repeats [32], or a highly conserved epitope also in the C-repeats [33] induced protection in murine models of infection, suggesting that a vaccine need not include serotype specific epitopes. Dale, on the other hand, engineered a chimera that combined multiple peptides in a formulation with up to 23 different M protein partial sequences [34].

Relative to M protein the surface bound streptococcal C5a peptidase has only recently been considered a vaccine candidate. Experiments presented here extend previous studies by showing that parenteral immunization with C5a peptidase derived from either group A or group B streptococci will induce serotype independent protection in mice. The immune response to streptococcal skin infection has not been seriously investigated, nor have vaccine candidates, including the C5a peptidase, been tested for their capacity to limit skin infections in animal models. The boundary between serotypes associated with impetigo and pharyngitis is not absolute. The M49 strain CS101 employed here and in previous experiments [10] is a serotype known to be associated with skin infections and acute glomerulonephritis. These streptococci were cleared more rapidly from the nasopharynx by immunized mice, leaving open the possibility that immunization with SCP protein could also enhance clearance of streptococci from the skin and prevent or reduce the incidence of impetigo.

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